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DOI: <https://doi.org/10.1159/000081633>

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ZORA URL: <https://doi.org/10.5167/uzh-49566>

Journal Article

Published Version

Originally published at:

Savaskan, E; Löffler, K U; Meier, F; Müller-Spahn, F; Flammer, J; Meyer, P (2004). Immunohistochemical localization of angiotensin-converting enzyme, angiotensin II and AT1 receptor in human ocular tissues. *Ophthalmic Research*, 36(6):312-320.

DOI: <https://doi.org/10.1159/000081633>

Immunohistochemical Localization of Angiotensin-Converting Enzyme, Angiotensin II and AT1 Receptor in Human Ocular Tissues

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Key Words

Angiotensin II · Angiotensin-converting enzyme ·
AT1 receptor · Retina · Alzheimer's disease

Abstract

We investigated the immunohistochemical distribution of 3 components of the renin-angiotensin system (RAS), angiotensin-converting enzyme (ACE), angiotensin II (AngII) and AT1 receptor (AT1), in the human eye. ACE and AngII were localized to nonpigmented epithelial cells of the ciliary body, to endothelial and epithelial cells of the cornea, to epithelial cells of the conjunctiva and to trabecular meshwork cells in the anterior part of the eye. In the posterior part of the eye, ACE and AngII were localized to ganglion cells, some cells in the inner nuclear layer, photoreceptor cells and to endothelial cells of the retinal and choroidal vessels. The overall intensity of AT1 immunoreactivity was weak in all ocular tissues, but the main localization was in ganglion cells. As a preliminary investigation, we were able to include 2 Alzheimer's disease (AD) cases. In AD, no differences from controls were found in the cellular distribution and staining intensity of all 3 antigens. The manifold localization sites of the observed antigens point to rather generalized func-

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Introduction

The renin-angiotensin system (RAS) is mainly implicated in the regulation of fluid homeostasis and blood pressure. Angiotensin II (AngII) is the vasoactive peptide generated from the inactive peptide angiotensin I by the enzymatic action of angiotensin-converting enzyme (ACE) [1–3]. Angiotensin I, on the other hand, is generated by the enzyme renin acting on the substrate angiotensinogen. The first evidence that the CNS may have its own endogenous RAS demonstrated the presence of precursor peptides and synthesizing enzyme ACE in the canine brain [4–6]. It is now well known that all components of the RAS exist in the brain [7]. Besides its action as a vasoactive peptide, AngII, as the active component of the RAS, exerts modulatory actions on neurons in the CNS [8, 9].

The immunohistochemical localizations of AngII and ACE in different brain regions has been well studied in rats [10–12] and humans [8, 9, 13], in particular the corti-

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0030-3747/04/0366-0312\$21.00/0

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cal distribution of the AT1 receptor (AT1), a 7-transmembrane receptor coupled to phospholipase C, calcium and cAMP second-messenger systems, which mediates most of the actions of AngII including blood pressure regulation and growth-promoting effects [2, 8, 14, 15].

Ocular structures which belong to the CNS have their own intraocular RAS independent of circulating RAS peptides [16, 17]. There is biochemical and immunohistochemical evidence for the localization of angiotensinogen, the precursor protein of angiotensins [18], AngII [19], ACE [17, 20] and AT1 [21, 22] in ocular neurons and vessels of rodents. In the human eye, angiotensin and ACE gene expressions have been identified, supporting the concept of intraocular synthesis of AngII, independent of precursor substances in the circulation [17]. Besides its vascular effects [23], the intraocular RAS has been postulated to have neuromodulatory functions, and, here, it may contribute to local pathologies such as proliferative retinopathy [16, 17].

The role of ocular RAS during the course of neurodegeneration has not been studied yet. Studies in Alzheimer's disease (AD) patients indicate a possible link between RAS activity and cognitive decline [3, 24]. Vascular factors resulting from RAS disturbances may precede AD [25]. Indeed, ACE activity has been found to be increased in the cortex of AD patients [1, 8, 26] parallel to a decrease in the cerebrospinal fluid levels [27]. Although contrary results exist, most of the case-control studies indicate that ACE gene polymorphism may provide a genetic susceptibility factor for AD [24, 28, 29]. The aim of the present study was to explore the morphological localizations of ACE, AngII and AT1 in human ocular tissues and to reveal possible alterations of the RAS in AD with immunohistochemistry.

Materials and Methods

Paraffin-embedded human ocular samples were used. The non-opened eye globes were fixed in 4% paraformaldehyde for 2 days and cut in the sagittal plane into 4- μ m-thick consecutive sections including peripheral, central and macular regions. The study consisted of 9 controls and 2 AD cases (table 1). The mean postmortem delay was 23 h 57 min for controls and 16 h 15 min for AD cases (table 1). The diagnosis of AD was made with clinical evaluation and confirmed by postmortem neuropathological examination. In most cases, the cause of death was heart failure or pneumonia. The sample collection was approved by the Ethics Committee criteria and according to the Helsinki Declaration of 1975.

The antigens were visualized by peroxidase staining using the substrate 3-amino-9-ethylcarbazole according to the manufacturer's instructions (Vector Laboratories). The method for immunohistochemistry has been described in detail elsewhere [30]. The primary

Table 1. Data of control (C) and AD cases

Case	Clinical diagnosis	Age years	Gender	PMD	CD
1	C	85	M	31 h	heart failure
2	C	97	F	26 h	heart failure
3	C	91	F	24 h	heart failure
4	C	69	F	20 h	pancreatitis
5	C	76	F	9 h 40 min	heart failure
6	C	88	F	31 h	heart failure
7	C	76	F	9 h	pneumonia
8	C	92	M	24 h 32 min	heart failure
9	C	85	F	6 h 33 min	heart failure
1	AD	83	M	15 h	heart failure
2	AD	84	M	17 h 30 min	pneumonia

PMD = Postmortem delay; CD = cause of death.

antibodies used were a mouse monoclonal antibody against the N-terminal end of human ACE (Chemicon International, Temecula, Calif., USA), an affinity-purified rabbit polyclonal antibody raised against a peptide corresponding to the amino acids 306–359 of the AT1 of human origin (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) and a rabbit anti-AngII (human) serum (Phoenix Pharmaceuticals, Belmont, Calif., USA). The specificity of the antibodies has been ascertained by previous experiments [31, 32]. The optimal concentration of the primary antibody was experimentally determined to be 1:100 for all antibodies. The samples were counterstained with Mayer's hemalum. The experiments were performed in duplicate. Control samples were stained simultaneously following the same procedure as the test samples with the exception that the primary antibodies were omitted. All sections were assessed for localization and intensity of specific immunoreactivity on a semiquantitative scale of +/+++ by two blind observers (table 2).

Results

The results presented here localize ACE, AngII and AT1 immunoreactivities to different cellular and vascular structures within the human eye. For cytoarchitectural organization of ocular cells, we follow the detailed descriptions by Spencer [33]. For the clarity of the overview the anterior and posterior compartments will be discussed separately, and the results are summarized in table 2 according to regional distribution and staining intensity.

Anterior Part of the Eye

In the anterior part of the human eye, the cornea and ciliary body belong to the regions where notable amounts of immunoreactive structures were present (table 2). In

Table 2. Semiquantitative assessment of the intensity of AngII-, ACE- and AT1-immunoreactive structures in the human eye

		Anterior eye						Posterior eye					
		cornea			ciliary body			retina			choroidea		
		ACE	AT1	AngII	ACE	AT1	AngII	ACE	AT1	AngII	ACE	AT1	AngII
Controls	1	+	–	+	–	–	+	++	–	++	+	–	–
	2	+	+	++	++	–	+	++	+	++	+	–	–
	3	+	–	+	+	–	+	++	–	++	+	–	+
	4	++	+	+++	+	–	+	++	–	++	+	–	–
	5	+	+	++	+	–	+	+	–	+	+	–	–
	6	+	–	+	+	–	+	+	+	++	+	–	–
	7	+	–	+	–	–	+	+	–	+	–	–	–
	8	+	+	+	+	–	+	++	–	++	+	–	–
	9	++	+	++	+	–	+	++	–	++	–	–	+
AD	1	++	–	++	+	–	+	++	–	++	+	–	+
	2	++	–	++	–	–	+	+	–	+	+	–	–

– = No immunoreactivity; + = slight; ++ = moderate; +++ = high.

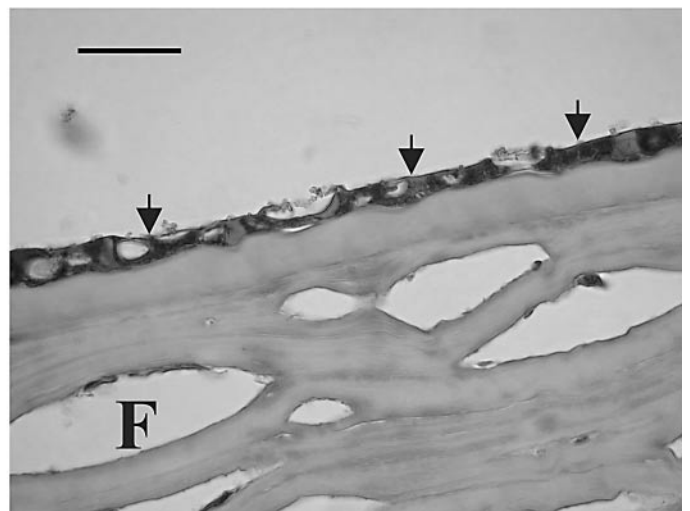
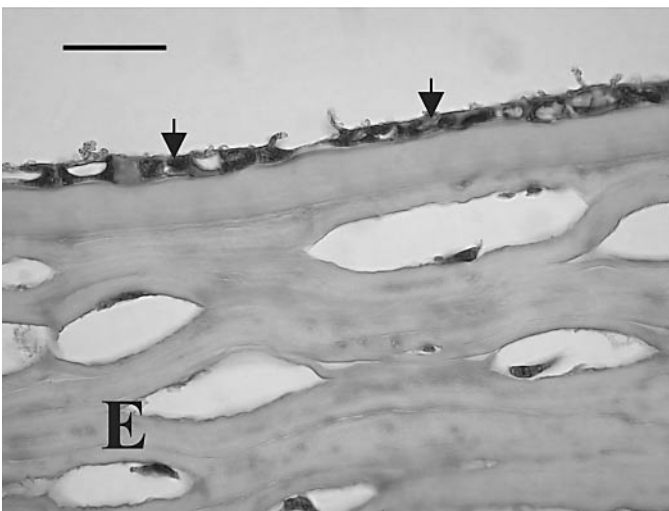
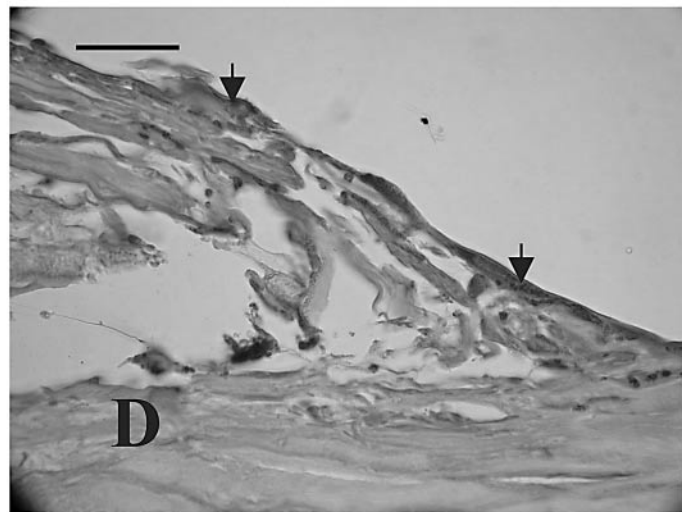
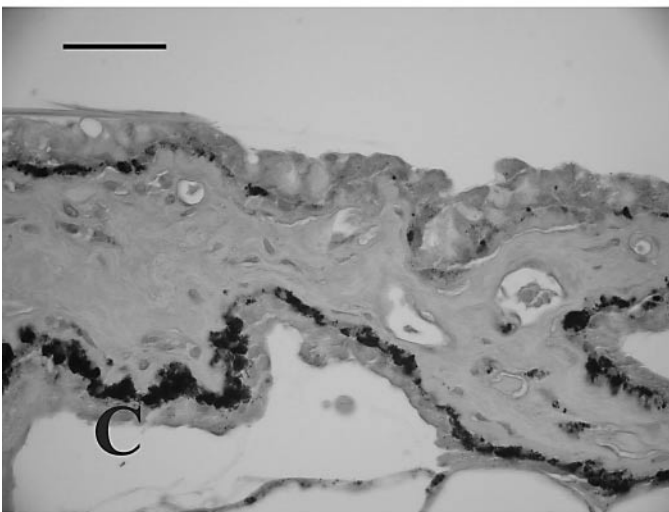
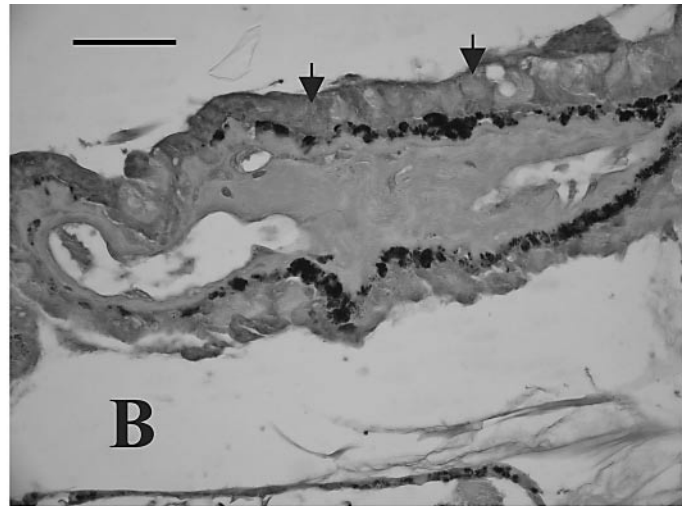
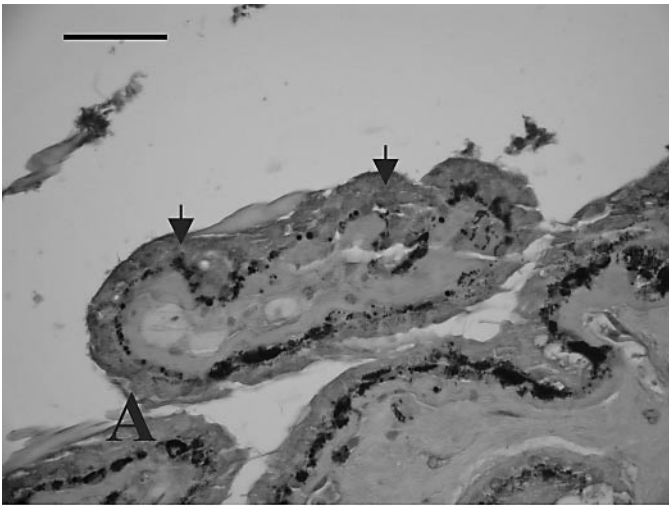
the ciliary body, nonpigmented epithelial cells presented intracellular staining for ACE and AngII (fig. 1A, B), whereas we could not observe any AT1 immunoreactivity in this cellular localization. Endothelial and epithelial cells of the cornea were distinctly immunoreactive for both ACE and AngII in some cases (fig. 1E, F). AT1 immunoreactivity was also present on the surface of these cells, but the overall intensity of the immunoreaction was weak, even missing in about half of the cases. In addition, single cells in the cornea, distinctly recognized as kerocytes, were immunolabeled for AngII. Both ACE and AngII immunoreactivities were also present in the epithelial cells of the conjunctiva where we were not able to detect AT1 labeling. Within the stroma of the iris single blood vessels were immunoreactive for ACE and AngII in their endothelial cells. Finally, trabecular meshwork cells were mostly labeled for both ACE and AngII (fig. 1D). The lens belonged to the regions displaying no staining for the RAS components. In AD cases, the cellular localizations and staining intensity for ACE, AngII and AT1 were comparable with the findings in controls (table 2).

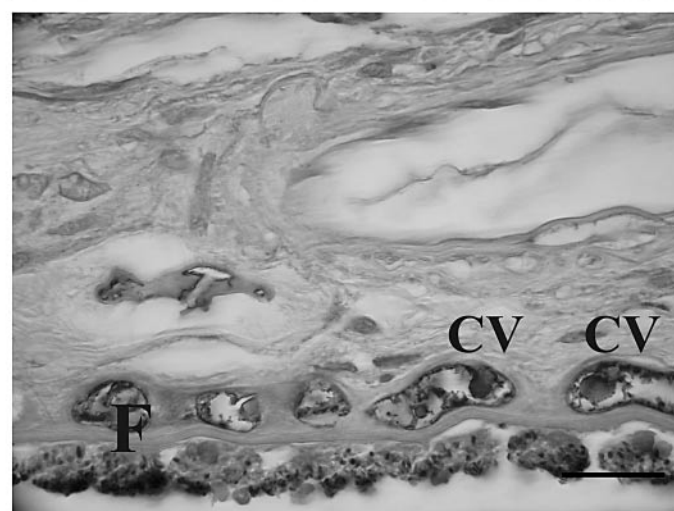
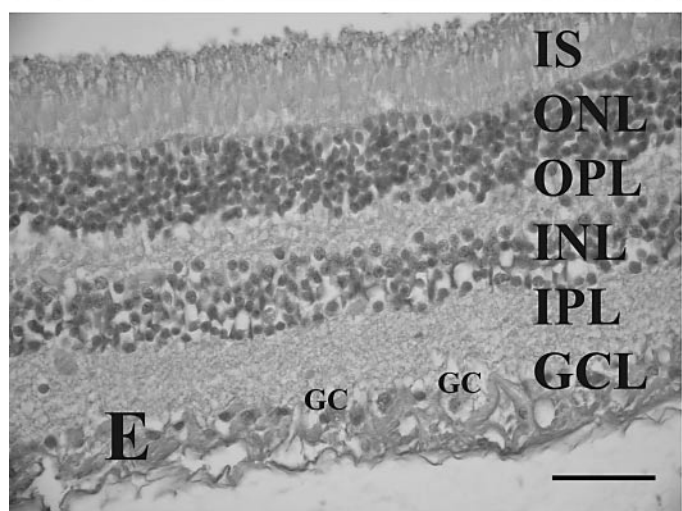
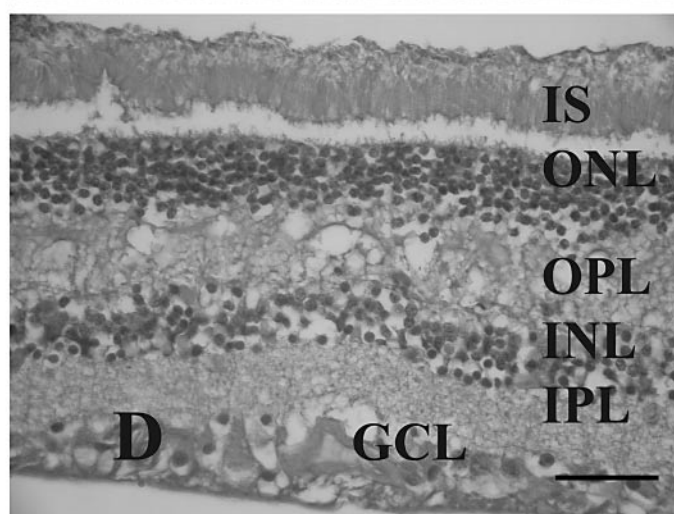
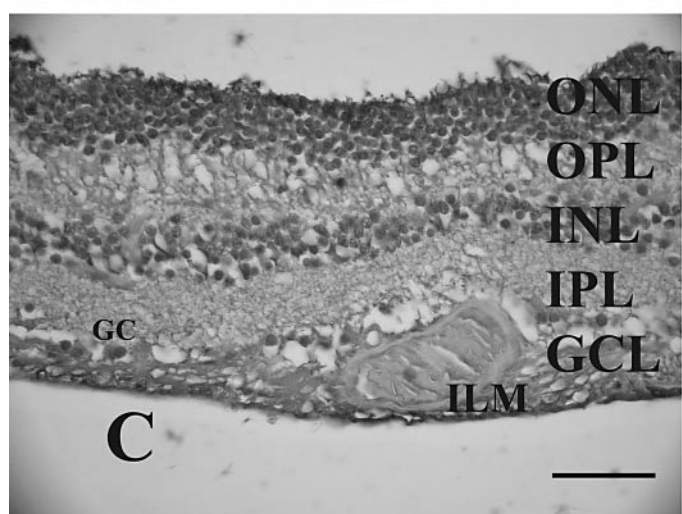
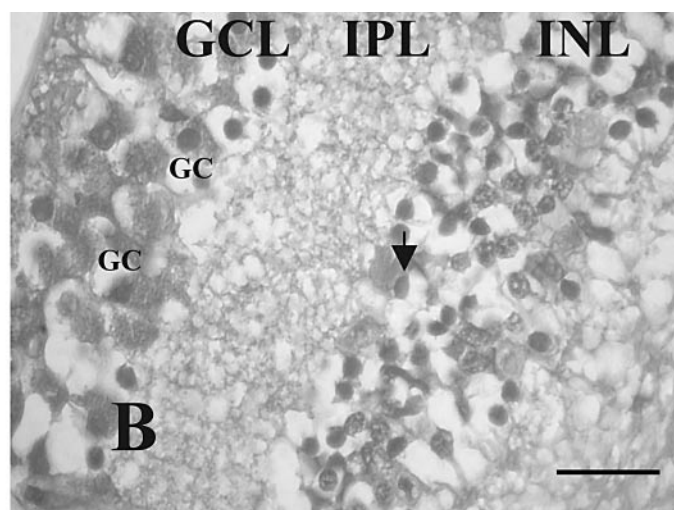
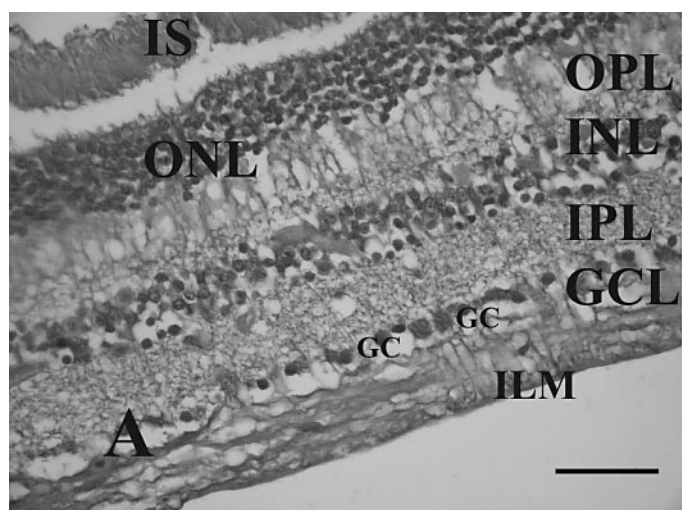
Posterior Part of the Eye

The retina revealed the most intense labeling for the RAS components ACE and AngII within the whole eye. The inner region of the limiting membrane was distinctly positive for ACE and AngII in its entire length (fig. 2A–C). In the ganglion cell layer (GCL), some of the ganglion

cells with round somata, 10–14 µm in diameter and with a vesicular nucleus, were stained for both ACE and AngII (fig. 2A–C), whereas the AT1 immunoreactivity was very weak and was found only in 2 controls on the cellular surface of the ganglion cells (table 2). The GCL contained also some ACE- and AngII-positive cellular processes. The endothelia of small vessels within the GCL were positive for ACE and AngII. Some immunoreactive cells (according to their shape probably bipolar cells) were scattered in the inner nucleiform layer (fig. 2B). In addition to the GCL and the inner plexiform layer, the outer plexiform layer contained cellular processes entirely stained for ACE and AngII. Both the outer nucleiform layer, containing the cell somata of the photoreceptor cells, and the inner segments of these cells were stained for ACE and AngII (fig. 2A–C).

Fig. 1. Sagittal sections through the anterior part of the human eye. ACE- (A) and AngII-immunoreactive (B) cells in the nonpigmented epithelium of the ciliary body as indicated by arrows. Low magnification (LM), scale bar = 50 µm. C Control section through the ciliary body stained simultaneously following the same procedure, with the exception that the primary antibody was omitted. LM. D AngII immunoreactivity in the trabecular meshwork (arrows). High magnification (HM), scale bar = 120 µm. ACE- (E) and AngII-immunoreactive (F) endothelial cells of the cornea as indicated by arrows. HM.





In the choroidea, endothelial cells of the large vessels displayed ACE and AngII immunoreactivity (fig. 2F), but no staining for AT1. The same was true for the central vessels within the optic nerve. Sclera contained no immunoreactive structures. As in the anterior part of the eye, there were no differences in cellular distribution and staining intensity of the observed immunoreactivities between controls and AD cases.

Discussion

The data presented here reveal the differentiated localization of the main RAS components in the human eye. The results support previous findings that ACE is present in ocular cells suggesting the local synthesis of AngII [17] and provide additional information on the cellular localization of AngII and its specific receptor AT1. In the aforementioned study, pooled tissue samples were used, and, using reverse-transcription polymerase chain reaction, gene expressions of renin, angiotensinogen and ACE were localized to the human retinal pigment epithelium, choroid and neural retina [17]. Angiotensinogen and ACE mRNAs were also found marginally in sclera samples. Our data complete these findings with the additional description of the RAS components in the anterior part of the eye and their immunohistochemical localization to distinct cells. Since 18 subjects with a mean age of 54 years and a range up to 78 years were included in the previous study in the human eye [17], the study samples are comparable with the elderly subjects in our study.

Fig. 2. Sagittal sections through the posterior part of the human eye. ILM = Inner limiting membrane; GCL = ganglion cell layer; GC = ganglion cells; IPL = inner plexiform layer; INL = inner nucleiform layer; OPL = outer plexiform layer; ONL = outer nucleiform layer consisting of cell somata of photoreceptor cells; IS = inner segments of photoreceptor cells; CV = choroidal vessels. LM = Low magnification (scale bar = 50 μ m); HM = high magnification (scale bar = 120 μ m). Low (**A**) and high (**B**) magnifications of ACE-immunoreactive structures in the retina. In **B**, ACE-immunoreactive ganglion cells are indicated by an arrow. **C** AngII-immunoreactive structures in the retina. LM. **D** AT1 immunoreactivity is very weak or even missing in the retina. LM. **E** Control section stained simultaneously following the same procedure, with the exception that the primary antibody was omitted. LM. **F** Choroidal vessels revealing ACE immunoreactivity in their endothelial cells. HM.

AngII acting within the ocular tissues has been suggested to be local in origin since its leakage from the circulation is minimal or even nonexistent, and both precursor and synthesizing enzymes are present within the eye [19]. The retinal pigment epithelium separates the retina from the choroid and forms, together with the pigment epithelium of the ciliary body and the endothelium of the retina vessels, the blood-retina barrier preventing the diffusion of circulatory RAS components [19, 34, 35]. AngII concentration in the vitreous humor has been found to be only about 10% of the plasma concentration, and in the retina and choroid it was 10 and 86 times higher, respectively, than in plasma [19] indicating a concentration of AngII synthesis in the outer part of the eye [17]. This interpretation is supported by the finding that a renin/prorenin gradient exists in vitreous humor, with the lowest levels present in the most anterior parts of the vitreous [36]. The previous study failed to show renin mRNA expression in the anterior part of the human eye [17]. However, in the bovine eye, renin has also been found in the anterior part [36]. Our data clearly show that ACE and AngII are present both in anterior and posterior parts of the eye supporting the hypothesis of local synthesis of AngII in the whole structure.

In the anterior part of the eye, ACE and AngII were mainly localized to endothelial and epithelial cells of the cornea and to nonpigmented cells of the ciliary body. The ciliary body is also the main localization for renin in the anterior part of the eye in a previous study in bovine ocular tissues [36]. Besides its expression in the posterior part of the eye, ACE mRNA has also been detected in the iris/ciliary body of the chicken eye [20]. It has been suggested that vasoconstriction in this well-vascularized area can be modulated by the local RAS, independently of the circulation-derived AngII and also independently of ACE synthesis by the other tissues [20], which is supported by our findings that both ACE and AngII are present in cellular localizations here. In addition to cellular structures, small vessels in the anterior area were positive for ACE. The effects of ACE inhibitors as highly effective antiglaucomatous substances lowering intraocular pressure [37] and preventing angiogenesis reducing the risk of diabetic proliferative retinopathy [38] may be explained by their actions on ocular vessels [20]. Since the epithelium of the ciliary body is the site of aqueous humor secretion [39], and the trabecular meshwork cells, which were immunopositive for both ACE and AngII in our series, are the main site of outflow facility [33], the ocular RAS in this region may be importantly involved in the regulation of aqueous humor outflow regulation.

The cellular localizations of ACE and AngII in the posterior part of the eye confirm previous findings in chicken [20, 40], rat [22], rabbit [19] and human [17] retina. In our series, the ACE- and AngII-immunoreactive cells in the GCL were clearly ganglion cells and the positive cells in the inner nucleiform layer may correspond to bipolar cells, probably amacrine or Müller cells. Similar to our results, ACE and AngII have been found localized to ganglion, amacrine, bipolar and photoreceptor cells in the retina [19, 20]. It has been suggested that amacrine cells account mainly for AngII synthesis in the retina [19] whereas ACE may be not an intrinsic enzyme of the AngII-positive amacrine cells but rather taken up from the intracellular space [20]. AngII in the retina may primarily act as a neurotransmitter or neuromodulator [16, 19]. Besides AngII, substance P has been identified as a target neuropeptide for the catabolic activities of ACE in the retina [41] emphasizing the neuromodulatory role of the RAS in this area. In particular, the ganglion cells were strongly immunoreactive for ACE and AngII in our study. The suprachiasmatic nucleus, the principal circadian pacemaker in mammals, receives efferent projections from the ganglion cells providing light information to entrain circadian rhythms [30, 42] and contains AngII-positive neurons [43] suggesting a possible neuromodulatory role for the RAS in this pathway.

In the present study, both ACE and AngII were localized to the inner segments of photoreceptor cells. ACE immunoreactivity in photoreceptor cells has also been previously described [19, 40], but, in contrast to the present study, these reports have failed to localize AngII in photoreceptor cells. It has been suggested that ACE may act predominantly as a general peptidase here. Our findings provide evidence for the presence of AngII in photoreceptor cells, but, since the mentioned previous studies were not performed in humans, we cannot exclude interspecies differences.

AT1 immunoreactivity was slight in ocular tissues. The detected immunoreaction was restricted to corneal cells in the anterior part of the eye and to ganglion cells in the retina. The overall intensity of the AT1 immunoreactivity was very weak, and, in the retina, it could be detected only in 2 controls. AT1 mRNA has been found in the rat retina, iris/ciliary body and choroid by reverse-transcription polymerase chain reaction [21, 22]. In only one study using immunohistochemistry AT1 has been localized to ganglion cells in the rat retina [22]. Since the same AT1 antibody was used in our and the latter study [22], species-specific differences may account for the discrepancy. However, taken together, the results identify

ganglion cells as a favored action site of AngII. This hypothesis is supported by the observation that AngII modulates the calcium concentration of retinal ganglion cells [44] emphasizing the role of AngII as a neuromodulatory substance.

ACE- and AngII-immunoreactive vessels were found both in the GCL and choroidea, but no AT1 immunoreactivity. This is in contrast with findings in the rat eye where AT1 has been found to be expressed in the uveal and epiretinal arteries, but not in the vessels of the inner retina leading to the suggestion that the neuronal retina may be not involved in vasoconstrictive functions [22]. Our data show that the RAS may be vasoactive both in the retina and choroidea. The blood supply of the human eye originates from the arteria ophthalmica but separates to two different sources, the arteria centralis retinae and choroidal vessels, supplying the retina and the choroidea separately [33]. The RAS may be effective in both vessel systems, but the vasoactive RAS components in the choroidea may be, in contrast to retinal ACE and AngII, of circulatory origin, since both parts are separated by a blood-retina barrier [19, 34, 35]. Besides its neuromodulatory actions, AngII in the retina has vascular growth-promoting effects, inducing neovascularization via angiotensin 2, and, therefore, plays a role in the progression of proliferative retinopathies in diabetes [45] extending the spectrum of RAS actions in the retina.

Neither the intensity nor the distribution of the observed immunoreactivities was altered in the 2 AD cases. In the CNS, findings are different: ACE density was increased in the cortex [1, 8, 26] and in the striatum [1] of AD patients parallel to an increased AngII immunoreactivity in the AD hippocampus and striatum [8]. Physiopathological hypotheses suggest a possible involvement of the RAS components in the course of AD [24]. Most of the performed case-control studies tend to suggest that the ACE genetic polymorphism may constitute a genetic susceptibility factor for AD [24] and RAS disturbances may contribute to the vascular pathology observed in AD [25]. Indeed, AngII immunoreactivity has been found to be increased around small vessels in AD striatum supporting the hypothesis of an underlying vascular pathology in AD [8]. Several reasons may account for the finding that the RAS components were not altered in the AD cases. First, only 2 AD cases could be included in the study, which precludes any generalization. Second, the diagnosis of the 2 AD cases confirmed with postmortem pathological examination excluded the possibility that these had a distinct vascular pathology. It may be that those dementia cases with more prominent vascular involvement are the

ones showing RAS alterations. Third, RAS alterations previously observed in AD were particularly localized in brain regions such as the hippocampus or cortex, which are highly and early implicated in AD pathology. RAS in ocular tissues may not have the same impact on AD

pathology as brain regions involved in higher cognitive functions. Nevertheless, the present data confirm that ocular RAS alterations may contribute to pathological changes of ocular fluid homeostasis, angiogenesis and local neuromodulatory actions.

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